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Identification of mutations in a candidate dengue 4 vaccine strain 341750 PDK20 and construction of a full-length cDNA clone of the PDK20 vaccine candidate☆

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ABSTRACT

Dengue 4 virus strain 341750 serially passaged 20 times in primary dog kidney (PDK) cells was shown to have reduced infectivity for rhesus monkeys but was immunogenic and protected the monkeys from challenge with low passage parent dengue 4 virus. The dengue 4 PDK20 virus was also shown to be attenuated for human volunteers. We compared the genomic nucleotide sequences of low passage parent and PDK20 attenuated vaccine strains and identified 11 nucleotide (nt) substitutions in the PDK20 genome. Five mutations caused amino acid changes in viral proteins E (N366N/S), NS1 (E146Q), NS4B (S/L112L and A240V), and NS5 (F/L790L). Silent mutations occurred in genes encoding NS1 (nt 2609), NS3 (nt 6113, 6230 and 6239) and NS5 (nt 8081 and 8588). A full-length cDNA clone of the dengue 4 strain 341750 PDK20 was constructed and RNA transcripts of the clone were infectious in monkey kidney (LLC-MK_2) and *Aedes albopictus* (C6/36) cells. The sequence analysis and availability of an infectious clone provide molecular tools to investigate the basis for the attenuation of dengue 4 virus.

1. Introduction

Dengue viruses belong to the *flavivirus* genus in the *Flaviviridae* family, and are endemic to tropical and subtropical areas of the world that are inhabited by the *Aedes* mosquito vector placing an estimated 2.5 billion people at risk [1]. The four distinct but closely related virus serotypes (dengue 1, -2, -3 and -4) cause a spectrum of human illness ranging from a mild flu-like syndrome with rash called dengue fever to severe and sometimes fatal dengue hemorrhagic fever/dengue shock syndrome, which is more common among infants and young children [2]. A primary infection with one serotype appears to confer life-long immunity against re-infection with the same serotype, but does not produce long-lasting cross-protective immunity against other serotypes. Dengue hemorrhagic fever occurs most frequently in patients experiencing a secondary dengue infection, and one mechanism proposed to explain this phenomenon is antibody dependent enhancement of infection, in which antibodies developed against the first infecting serotype virus enhance a later infection with a different serotype [3]. Other factors proposed to contribute to the disease process include infecting strain, virus burden and cross-reactive T lymphocytes [4–6]. Because of the association of dengue hemorrhagic fever with secondary dengue infections, safe and effective vaccines that provide simultaneous protection against all four dengue virus serotypes are sought. A greater understanding of the molecular genetics of dengue virulence is also a high priority.

The *flavivirus* genome consists of single-stranded positive sense RNA approximately 11 kb long that is capped at the 5′ end and lacks a 3′ polyadenylated tail. The genome encodes a single long open reading frame flanked by 5′ and 3′ non-coding regions of variable size depending on the flavivirus. Three structural proteins, capsid (C), premembrane (prM), and envelope (E), and seven nonstructural (NS) proteins are co- and post-translationally cleaved from the polyprotein precursor by viral and cellular proteases, and occur in the polyprotein in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 [7,8].

Live-attenuated viruses are widely used for the prevention of human viral diseases. Attenuation of virulent wild viruses has gen-

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14. ABSTRACT

Dengue 4 virus strain 341750 serially passaged 20 times in primary dog kidney (PDK) cells was shown to have reduced infectivity for rhesus monkeys but was immunogenic and protected the monkeys from challenge with low passage parent dengue 4 virus. The dengue 4 PDK20 virus was also shown to be attenuated for human volunteers. We compared the genomic nucleotide sequences of low passage parent and PDK20 attenuated vaccine strains and identified 11 nucleotide (nt) substitutions in the PDK20 genome. Five mutations caused amino acid changes in viral proteins E (N366N/S). NS1 (E146Q). NS4B (S/L112Land A240V). and NS5 (F/L790L). Silent mutations occurred in genes encoding NS1 (nt 2609), NS3 (nt 6113 6230 and 6239) and NS5 (nt 8081 and 8588). A full-length eDNA clone of the dengue 4 strain 341750 PDK20 was constructed and RNA transcripts of the clone were infectious in monkey kidney (LLC-MK2) and Aedes albopictus (C6/36) cells. The sequence analysis and availability of an infectious clone provide molecular tools to investigate the basis for the attenuation of dengue 4 virus.

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erally been accomplished by serial passage of virus in cells from non-natural hosts. To develop a live-attenuated vaccine, low passage dengue 4 virus strain 341750 was serially passaged in primary dog kidney (PDK) cells and tested in monkeys [9]. By the 15th PDK passage, the virus was less infectious for monkeys compared to the low passage parent virus but remained immunogenic. The 30th PDK passage virus was non-infectious for monkeys. A vaccine lot was prepared from the 20th PDK passage by amplification in primary fetal rhesus lung cells to increase the titer, and was shown to protect vaccinated rhesus monkeys from challenge with the homologous parent dengue 4 strain and a heterologous dengue 4 strain H241 [9]. The dengue 4 PDK20 vaccine was shown to produce mild symptoms in human volunteers, and five of eight (63%) volunteers developed anti-dengue neutralizing antibody responses [10]. The PDK20 candidate was therefore selected for further clinical trials [11]. The aim of the present study was to identify the nucleotide and amino acid changes that occurred in the dengue 4 virus during progressive attenuation and also to construct a full-length infectious cDNA clone of dengue 4 vaccine strain PDK20.

2. Materials and methods

2.1. Viruses

Dengue 4 virus strain 341750 was isolated in 1982 from a patient in Colombia, South America by one passage in mosquitoes [12]. Virus was passaged 5 times in primary green monkey cells and 4 times in fetal rhesus lung (FRhL) cells to become the low passage parent strain and was passaged 6 and 20 times in PDK cells to become PDK6 and PDK20 attenuated vaccine strains. Each was then amplified three (PDK6) or four (PDK20) times in FRhL cells to make vaccine [12].

2.2. Cells

Vero and LLC-MK_2 cells were grown at 37 °C in a humidified incubator under 5% CO_2 in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 50 μg of gentamicin sulphate per ml of culture medium. *Aedes albopictus* C6/36 mosquito cells were grown at 28 °C in the same medium supplemented with 1× nonessential amino acids, 0.1 mM sodium pyruvate and 25 mM HEPES, pH 7.5.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Dengue 4 strain 341750 low passage parent, PDK6 and PDK20 virus strains were provided in lyophilized form by Ken Eckels, Walter Reed Army Institute of Research. Virus was suspended in 1.0 ml sterile water, RNA was prepared using a QiaAmp viral RNA kit (Qiagen) and an aliquot of RNA was combined with 50 pmol 3′ end primer (Table 1) and denatured for 3 min at 72 °C and chilled on ice. For reverse transcription, the RNA–primer mixture was added to a 100 μl reaction mixture that contained 1× RT buffer, 0.5 mM each deoxynucleotide triphosphate (dNTP), 10 mM DTT and 40 U RNasin RNase inhibitor (Promega). This mixture was divided into halves; to one 50 U Superscript II reverse transcriptase was added and the other served as a negative control. Both reactions were incubated for 1–2 h at 40 °C and terminated after incubation for 5 min at 95 °C. To amplify DNA by the polymerase chain reaction (PCR), 1 μl cDNA was added to a 49 μl reaction mixture that contained 1× PCR buffer, 0.2 mM each dNTP, 25–50 pmol each PCR primer, and either 2.5 U of high-fidelity *Pfu* DNA polymerase (Stratagene) to produce three overlapping DNA fragments for assembling the infectious clone, or 2.5 U Expand DNA polymerase (Boehringer Mannheim) to produce six overlapping DNA fragments for sequence analysis. Primers are given in Table 1. The PCR mixture was subjected to denaturation at 94 °C for 5 min followed by 35 temperature cycles each being 94 °C for 10 s, 45 °C for 30 s and either 68 °C (Expand) or 72 °C (*Pfu*) for 6 min. Products were visualized by 0.8% agarose gel electrophoresis.

Table 1
Forward (F) and reverse (R) primers used to amplify the dengue 4 genome.

Primer	Gene	Sequence
1F[a]	5′NCR	5′-AGAACCTGTTGGATCAACAACACCAAT-3′
2235F[b]	E	5′-GGAAAGGCTGTGCACCAGGTTTTTGGA-3′
3040F	NS1	5′-AAAACCAGACCTGGCAGATAGAGAAAG-3′
5057F	NS3	5′-GGATGAGGACATTTTTCGAAAGAAAAG-3′
7331F	NS4B	5′-CATGCTACTAGTCTTGTGTGCTGGACA-3′
8568F	NS5	5′-CCAATGGTGACTCAGTTAGCCATGACA-3′
2357R	E	5′-TGAAGTGTTTGAGTTCGTGCCAATCCA-3′
3484R	NS2A	5′-TGTCCGGCCGTCACCTGTGATTTGACC-3′
3853R	NS2A	5′-CAGTGATATTCCATCAATGAGTTCCAT-3′
5339R	NS3	5′-TGATGACAAAAGTCTTGTTGTGAAGGGT-3′
7523R	NS4B	5′-CAGTCCAGCTCCCGCCAAGTAACTTCC-3′
8841R	NS5	5′-TCACAGTTCACTGGCTGATGTCCATCC-3′
9771R	NS5	5′-CTATCAGTTCATCCTGGTTTCTACATGG-3′
10649R	3′NCR	5′-AGTTGTTAGTCTGTGTGGACCGACAAG-3′
97R[c]	5′NCR	5′-CCAGAGATCTGCTCTCTATTCAAACAAAC-3′
10590F	3′NCR	5′-ACATCAATCCAGGCACAGAGCGCCGCA-3′

[a] Primer pairs 1F-3484R, 3040F-8841R, 7331F-10649R were used to amplify three overlapping fragments to construct the dengue 4 infectious clone. Primer 10649R was used to initiate reverse transcription of purified genomic RNA.
[b] Primers pairs 1F-2357R, 2235F-3853R, 3040F-5339R, 5057F-7523R, 7331F-9771R and 8568F-10649R were used to amplify overlapping DNA fragments to derive the genome sequence.
[c] Primer 97R was used to initiate reverse transcription of the RNA genome after its circularization and primer pair 10590F-97R amplified the fragment encoding the 5′–3′ junction.

2.4. DNA sequencing

Oligonucleotide primers were designed from a published sequence of dengue 4 strain 814669 (GenBank accession number M14931). To obtain 5′ and 3′ termini DNA for sequence analysis, the genome was circularized to form a junction which served as a template for RT-PCR [13]. First, the 5′ cap structure was removed by treating genomic RNA with 5 U tobacco acid pyrophosphatase in 1× buffer (Epicenter), 40 U RNAsin and 0.2 mM ATP in a 20 μl reaction volume for 1 h at 37 °C. Decapped RNA was extracted once with Aqua-Phenol (Ambion) and once with chloroform, after which RNA was precipitated with 0.3 M sodium acetate/ethanol. The RNA was resuspended in water and ligated by incubation at room temperature for 3 h in a 20 μl reaction mixture containing 5 U T4 RNA ligase and 1× ligase buffer (New England Biolabs), 10% DMSO, 1 mM ATP and 40 U RNAsin. Ligated RNA templates were reverse transcribed to cDNA using a 5′-end reverse primer and amplified by PCR using 3′-end forward and 5′-end reverse primers (Table 1).

Prior to starting all sequence reactions, unincorporated PCR primers were removed by incubating the PCR product with 2 μl Exozapit reagent (Boehringer Mannheim) for 15 min at 37 °C followed by heat-inactivation of the reaction mixture for 15 min at 80 °C. Sequence analyses of PCR products were performed on an ABI PRISM 3100 Genetic Analyzer using the ABI version 3.1 sequencing kit (Applied Biosystems). Each 20 μl sequence reaction contained 20 ng PCR product or 200 ng plasmid, 5–10 pmol primer and 7 μl sequence kit reagent. Sequence analysis was performed using Sequencher software (Gene Codes Corp).

2.5. Plaque assay

Virus titers were determined by quantitative plaque assay of virus on LLC-MK_2 cells in six-well plates. Serial dilutions of virus were adsorbed to cell monolayers for 1.5 h at room temperature after which the cells were overlaid with 3 ml of melted 1.0% SeaPlaque agarose (FMC Bioproducts) in Earle's balanced salt solution without phenol red and with 0.357% sodium bicarbonate, 10.0%

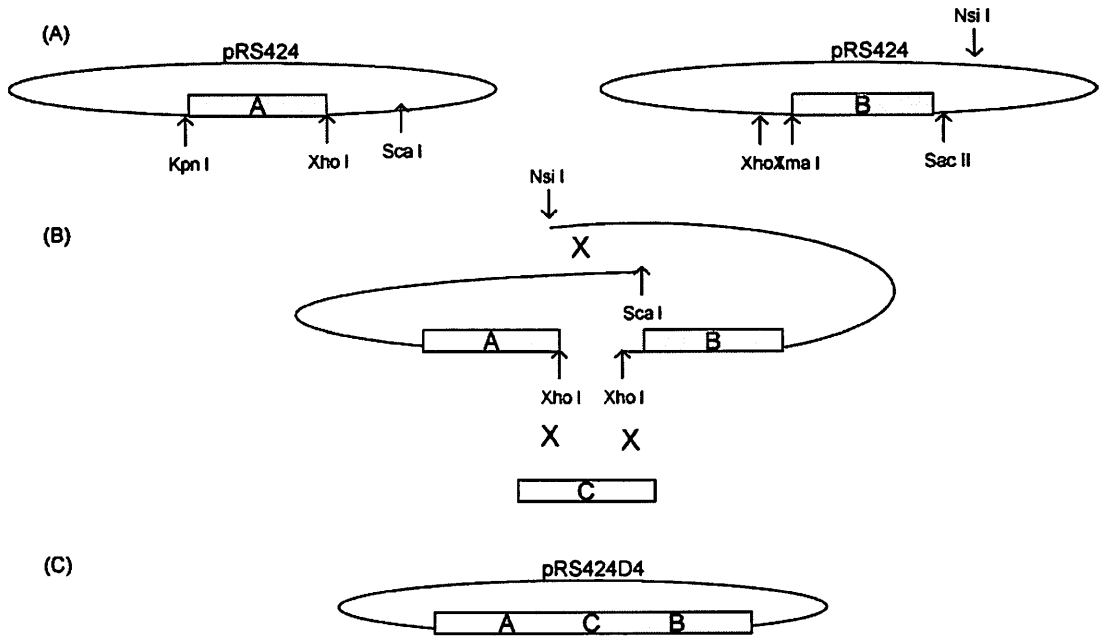

Fig. 1. Construction of a full-length cDNA clone of dengue 4 strain 341750. Three overlapping dengue 4 PDK20 DNA fragments, A (nt 1–3484), B (7331–10723) and C (3040–8841) were amplified by RT-PCR. (A) Restriction enzyme sites added to fragments A and B by PCR primers were 5′ KpnI and 3′ XhoI, fragment A, and 5′ XmaI and 3′ SacII, fragment B. Each fragment was inserted into the multiple cloning site of yeast–*E. coli* shuttle vector pRS424 to yield plasmids pRS424A and pRS424B. (B) pRS424A was digested with XhoI and ScaI and pRS424B with XhoI and NsiI, and together with fragment C were transformed into yeast strainYPH857. (C) Full-length clone resulting from triple homologous recombination in yeast. Recombinants were selected by PCR-screening for junctions between A and C and B and C.

FBS, and 1× each glutamine, MEM vitamins and amino acids (all from Invitrogen) and incubated at 37 °C. After 6 days, plaques were stained with a second layer of 3 ml of 1% SeaPlaque agarose containing 0.4 mg neutral red per ml, and plates were further incubated at 37 °C overnight. Virus plaques were counted the following day.

2.6. Radiolabeling and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein expression

LLC-MK_2 cells in duplicate wells of a six-well plate were mock-infected or infected at a multiplicity of infection (MOI) of 0.01 plaque forming units (PFU) with dengue 4 PDK20 virus or with transcript-derived virus from infectious clones. After 5 days, media was exchanged with methionine-free media and cells were starved for 1 h, and then labeled with 100 μCi ^{35}S-methionine plus cysteine for 2 h. Cell lysates prepared in radioimmunoprecipitation assay (RIPA) buffer were precipitated with dengue 4 polyclonal hyperimmune mouse ascitic fluid and analyzed by SDS-PAGE using ^{14}C-labeled protein markers to determine molecular weights [14]. The RIPA buffer consisted of 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 100 mM Tris pH 7.5 and 300 mM sodium chloride.

2.7. Growth kinetics

Subconfluent monolayer cultures of LLC-MK_2 and C6/36 cells growing in 25 cm^2 flasks were infected with dengue 4 PDK20 vaccine and infectious clone-derived viruses at a MOI of 0.01 for LLC-MK_2 cells and 0.0025 for C6/36 cells. Aliquots of culture fluid were removed daily for 10 days and stored at −70 °C. The titer of dengue virus in each sample was determined by plaque assay titration on LLC-MK_2 cells.

2.8. Transformation of yeast and Escherichia coli

Yeast strain *Saccharomyces cerevisae* YPH857 was originally a gift from Forrest Spencer (Johns Hopkins University, Baltimore, MD). Yeast were made competent by incubating log phase cells for 10 min in buffer containing 1 M Sorbitol, 10 mM bicine pH 8.35, 3% (w/v) polyethyleneglycol-1000 [15]. For homologous recombination experiments, competent yeast were incubated with 1–2 μg DNA in a 500 μl reaction volume containing 40% (w/v) polyethylene glycol-1000 buffered with 200 mM bicine pH 8.35 for 1 h at 30 °C. Following incubation, cells were pelleted by centrifugation, washed once with buffer containing 150 mM sodium chloride and 10 mM bicine pH 8.35, resuspended in the same buffer, and spread on standard minimal yeast media plates which lacked tryptophan to select for transformants. Yeast DNA was prepared as described and transformed into chemically competent *E. coli* STBL2 cells to amplify plasmid DNA [15].

2.9. Construction of a full-length cDNA clone of dengue 4 PDK20 virus

To construct a full-length cDNA clone, viral RNA from dengue 4 PDK20 was prepared and reverse-transcribed as described, and three overlapping fragments A, B and C were amplified using high-fidelity *Pfu* DNA polymerase. The PCR primers incorporated unique restriction sites that flanked fragments A and B, so that each fragment could be cloned into the multiple cloning site of a separate yeast–*E. coli* pRS424 shuttle vector, generating recombinant plasmids pRS424A and pRS424B (Fig. 1). An equal 1 μg amount of pRS424A, pRS424B and fragment C, the ends of which overlapped the ends of fragments A and B, was transformed into yeast. In yeast, triple homologous recombination produced the desired full-length clone. The clone was amplified in *E. coli* STBL2 cells as described [16].

To test for infectivity, 2 μg clone DNA was linearized immediately adjacent to the 3′ end of the genome using a unique a SacII site in the pRS424 plasmid vector. RNA transcripts were synthesized in 30 μl reaction mixtures containing the linearized DNA, 0.5 mM each rATP, rCTP, and rUTP, 0.1 mM rGTP, 0.5 mM cap analog m7G(5′)ppp(5′)G (New England Biolabs), 10 mM DTT, 40 U RNasin,

30 U SP6 RNA polymerase and 1× SP6 buffer (Promega). Reaction mixtures were incubated at 40 °C for 1 h and an aliquot of RNA was examined by 0.8% agarose gel electrophoresis. One half of the RNA produced was transfected into LLC-MK_2 cells by electroporation. For this, approximately 2×10^6 cells were washed and resuspended in 0.3 ml phosphate-buffered saline and mixed with RNA in a 0.4-cm gap electroporation cuvette (Biorad). The suspension was chilled on ice for 10 min and then pulsed at 200 V and 950 μF, placed on ice for a few seconds, and resuspended in 3.0 ml growth medium and seeded into 25 cm^2 tissue culture flasks. The next day, cell culture media was replaced with fresh media and after 2 days cells were trypsinized and an aliquot was seeded to a 1 cm^2 chamber on a slide (Nunc). The following day, media was removed and cells were rinsed twice with phosphate-buffered saline, air-dried, fixed and permeabilized in ice cold acetone for 2 min, air-dried and visualized by indirect immunofluorescence using dengue 2 anti-NS1 monoclonal antibody 7E11 as primary antibody followed by secondary FITC-labeled goat anti-mouse antibody.

3. Results

3.1. Nucleic acid sequence analysis

Complete genomic nucleotide sequences (10,649 nucleotides) of dengue 4 strain 341750 low passage vaccine parent and live-attenuated PDK6 and PDK20 viral genomes were determined, Genbank accession number GU289913. The analysis used RT-PCR products and was therefore considered to yield a population average sequence. Eleven mutations distinguished the PDK20 strain from its low passage parent virus (Table 2). Five mutations caused amino acid changes in E (N366N/S), NS1 (E146Q), NS4B (S/L112L and A240V) and NS5 (F/L790L). The remaining six mutations were silent and occurred in genes encoding NS1 (nt 2609), NS3 (nt 6116, 6230 and 6239), and NS5 (nt 8081 and 8588). The vaccine candidates that we sequenced are WRAIR pre-transfection vaccine candidates and these sequences may not be identical to the unpublished sequence of the current WRAIR/GlaxoSmithKline vaccine candidate.

The PDK6 intermediate passage level strain was different from both low passage parent and PDK20 viruses at three positions. Residue 383 in E is a single amino acid R in PDK6 but a G/R mixture in parent and PDK20 viruses. Residue 240 in NS4B is a mixture of amino acids A/V in PDK6 but is A in parent and V in PDK20. The third change is a silent mutation at nucleotide 8081 in the NS5 gene which is C/T in PDK6, T in low passage parent and C in PDK20. The PDK6 virus was identical to the PDK20 virus and different from low passage parent in NS5 with amino acid change L/F790L, and in NS3 at nucleotide 6239, which was a silent change of A/T to T change. Otherwise, low passage parent and PDK6 sequences were the same.

Table 2
Nucleotide mutations and deduced amino acid changes among dengue 4 strain 341750 low passage vaccine parent, PDK6 and PDK20 viruses.

Nucleotide	Parent	PDK6	PDK20	Gene	AA change[a]
2035	A	A	A/G	E	N366N/S[a]
2085	A/G	A	A/G	E	383[b]
2609	G	G	A	NS1	Silent
2859	G	G	C	NS1	E146Q
6116	G	G	A	NS3	Silent
6230	T	T	T/A	E	Silent
6239	A/T	A	A	NS3	Silent
7162	C	C/T	T	NS4B	S/L112L
7546	C	C/T	T	NS4B	A240V
8081	T	C/T	C	NS5	Silent
8588	C/T	C/T	C	NS5	Silent
9927	T/C	C	C	NS5	F/L790L

[a] Amino acid change between low passage parent and PDK20 is given as the amino acid found in parent virus followed by the residue number in the individual protein followed by the amino acid in PDK20. For example, N366N/S is a change at E protein residue 366 from a single asparagine to a mixture of asparagine and serine. Mixtures of nucleotides or amino acids are indicated by a slash between the two components.
[b] For parent and PDK20 viruses, E protein amino acid position 383 has a G/R mixture but is a single R in the PDK6 virus.

3.2. Production of the infectious clone virus

Three overlapping DNA fragments (A, B and C) were PCR-amplified from dengue 4 PDK20 cDNA using *Pfu* DNA polymerase and assembled into a full-length clone by homologous recombination in yeast using yeast–*E. coli* shuttle vector pRS424 (Fig. 1). Dengue 4-specific primers were made that incorporated enzyme restriction sites KpnI and XhoI to flank fragment A (nt 1–3484) and XmaI and SacII to flank fragment B (nt 7331–10,649), as shown in Fig. 1. Fragments A and B were digested with appropriate enzymes to cut the flanking sites (KpnI and XhoI for fragment A and XmaI and SacII for fragment B) and each fragment was ligated into the corresponding multiple cloning site of a pRS424 vector to generate pRSDEN4A and pRSDEN4B (Fig. 1A). Recombinant vector pRS424A was digested with XhoI and ScaI and pRS424B with XhoI and NsiI, and both vectors together with fragment C were transformed into yeast strain YPH857 (Fig. 1B). Yeast colonies were selected using solid media lacking tryptophan. Triple homologous recombination between A, C and B (Fig. 1C) was confirmed by PCR amplification of DNA corresponding to the junctions between A and C and B and C. Two recombinant yeast colonies determined to be positive for the triple recombination were selected for DNA purification and transformation of STBL2 cells. A plasmid clone from each yeast DNA was prepared from bacteria, termed clones 1 and 2.

Linearized plasmid DNA from clones 1 and 2 served as templates to produce full-length RNA transcripts for transfection of LLC-MK_2 cells by electroporation. Cells were analyzed by an indirect immunofluorescence assay 6 days post-transfection using anti-NS1 monoclonal antibody 7E11 and determined to be infectious by detection of NS1 protein expression (Fig. 2). High-titer stock virus was produced by infecting C6/36 cells with electroporation supernatants and harvesting the C6/36 supernatant after 7 days. Titers of transcript-derived viruses from clones 1 and 2 were 5.5×10^6 PFU/ml and 1.6×10^6 PFU/ml, respectively, which are expected titers for dengue 4 virus grown in C6/36 cells. The plaque phenotype of the PDK20 virus and clones 1 and 2 viruses was identical.

The sequence of the complete dengue genome in clone 1 was found to differ from the PDK20 vaccine virus at two positions resulting in a L284F substitution in E and a silent A–G mutation of nucleotide 5759 in NS3.

3.3. Characterization of dengue 4 PDK20 infectious clone virus

The pattern of dengue 4 protein synthesis in cells infected with PDK20 vaccine virus was compared to the pattern produced by clone-derived virus. Radiolabeled cell extracts prepared from mock-infected cells or cells infected with PDK20 vaccine virus or PDK20 clone-derived virus were immunoprecipitated with polyclonal dengue 4-specific antibodies and analyzed by SDS-PAGE and fluorography [14]. Dengue 4 proteins NS2B, prM, NS1, E and NS3 bands were identified on the basis of mobility (Fig. 3). The pattern of dengue 4-specific protein synthesis is identical for vaccine and clone-produced viruses.

Growth kinetics of clone-derived viruses was compared to that of the PDK20 vaccine virus in LLC-MK_2 and C6/36 cells (Fig. 4A and B). Cells were infected at the same MOI with each virus, and small aliquots of media were removed daily until day 10. Results showed that viruses produced from both clones grew at the same rate as

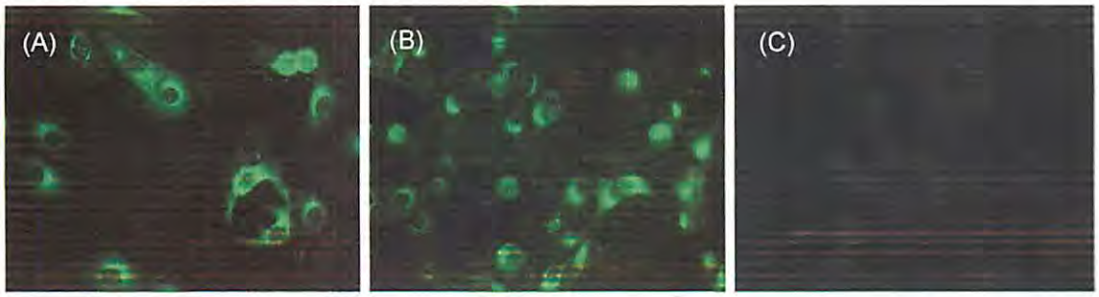

Fig. 2. Infectivity assay for dengue 4 PDK20 infectious clones. LLC-MK_2 cells transfected with RNA transcripts of (A) clone 1, (B) clone 2, or (C) no RNA were stained using anti-NS1 monoclonal antibody 7E11 as primary antibody and FITC-conjugated goat anti-mouse gamma globulin as secondary antibody.

PDK20 vaccine virus and that peak titers were 5×10^4 PFU/ml in LLC-MK_2 and 4×10^6 PFU/ml in C6/36 cells.

4. Discussion

Eleven nucleotide substitutions were found to distinguish the dengue 4 low passage parent virus from its PDK20 derivative (Table 2). Five of the mutations resulted in single amino acid substitutions in viral proteins NS1 (E146Q) and NS4B (A240V), a mixture of amino acids in E (N366N/S), and a change from a mixture to a single amino acid in NS4B (S/L112L) and NS5 (F/L790L). Silent mutations occurred in NS1 (nt 2609), NS3 (nt 6116, 6230 and 6239) and NS5 (nt 8081 and 8588). Most mutation sites in the PDK20 virus are unique to the dengue 4 strain and some are shared by the other WRAIR live-attenuated vaccine candidates (Fig. 5). An infectious clone of the dengue 4 PDK20 strain was produced and will be used in future studies to isolate and characterize individual mutations that may have contributed to the attenuation of the PDK20 virus. Knowledge of virulence and attenuation factors is important to the development of improved safe and effective flavivirus vaccines.

The single E mutation in the PDK20 virus changed a single polar residue to a mixture of polar and uncharged amino acids (N366N/S) at this position. The viral E protein is responsible for cell receptor binding and membrane fusion and it is the primary antigen inducing protective immunity. Three separate structural domains, central domain I, dimerization/fusion domain II, and receptor binding/antigenic domain III were assigned based on the crystal structure of a soluble E protein fragment from tick-borne encephalitis and dengue 2 and dengue 3 viruses [17–19]. A fourth domain, the C-terminal stem-anchor, was not part of crystallized proteins. Upon exposure to low pH in the endosome of infected cells, E protein dimers undergo a conformational change that rearranges E into trimers and exposes the domain II fusion peptide for fusion of the virion with the endosomal membrane [20,21]. This transformation involves relatively large spatial transitions at the interface between domains I and II, and at the interface between domains I and III [20–22]. The dengue 4 E protein N366N/S mutation is located within a region that interconnects domains I and III. Mutations within the interfaces between domains I and II and I and III are found in several flaviviruses that have been adapted to grow in an alternate host and are thought to influence virus pathogenicity [17,23–29]. A dengue 3 virus passaged in monkey kidney cells and intracerebrally in mice accumulated substitutions in the domain I/II interface, and these appeared to modulate fusion functions of the virus by altering the fusion pH threshold [25]. A mouse neurovir-

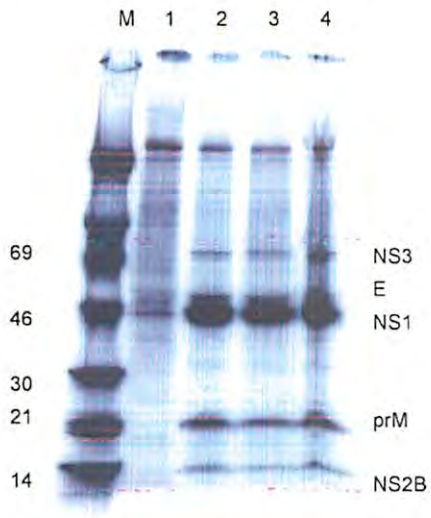

Fig. 3. Protein expression by transcript-derived virus. LLC-MK_2 cells were mock-infected (Lane 1) or infected with dengue 4 PDK20 virus (Lane 2) or transcript-derived virus from clones 1 and 2 (Lanes 3 and 4), labeled with (^{35}S)-methionine, lysed in RIPA buffer, immunoprecipitated with dengue 4 polyclonal hyperimmune ascites fluid and analyzed by SDS-PAGE. Lane M, ^{14}C-labeled protein markers, with molecular masses in kilo Daltons. The positions of E, prM, NS1, NS2B and NS3 proteins are indicated at the right side.

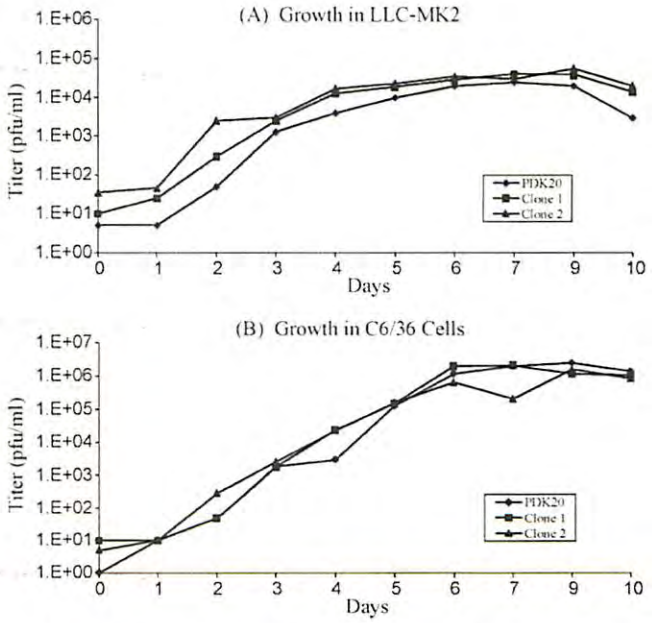

Fig. 4. Growth of transcript-derived virus versus dengue 4 PDK20. LLC-MK_2 cells were infected with an MOI of 0.01 (A) and C6/36 cells were infected with an MOI of 0.0025 (B) with dengue 4 PDK20 or with transcript-derived virus clones 1 and 2 or with virus transcribed from clones 1 and 2. An aliquot of culture media was removed daily, stored at −80 °C until use, and the virus titers were determined by plaque assay on LLC-MK_2 cells.

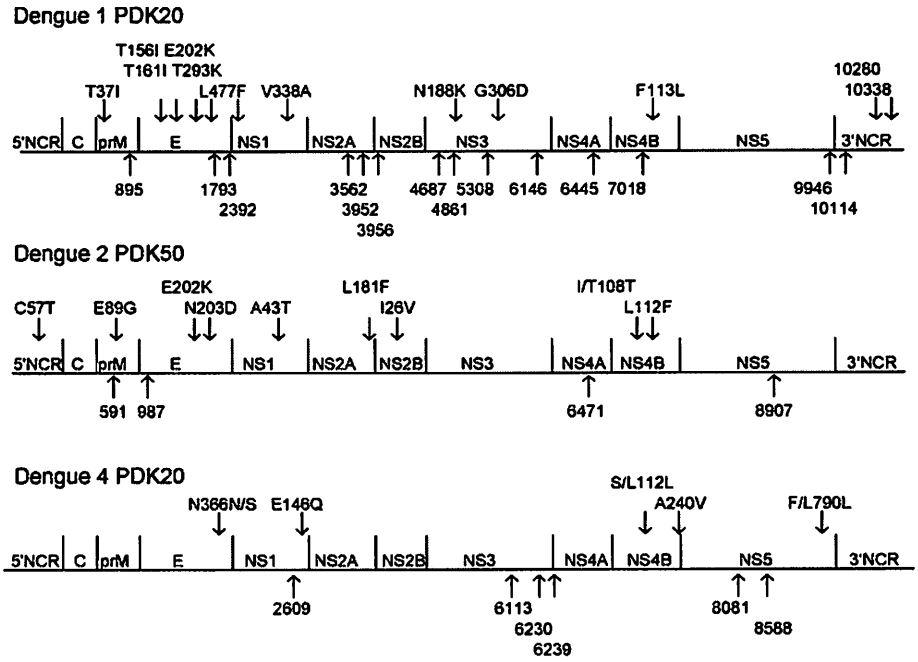

Fig. 5. Alignment of dengue 1, 2 and 4 genomes and locations of mutations in WRAIR live-attenuated vaccine strains dengue 1 PDK20, dengue 2 PDK50 and dengue 4 PDK20. Arrows above the genome line point to amino acid changes that are numbered according to location in the individual protein and nucleotide changes in the 5′ and 3′ NCRs, which are labeled by nucleotide number from the 5′ end. Arrows below the genome show locations of silent nucleotide mutations and are labeled by nucleotide number beginning from the 5′ end. Dengue-1 and -2 virus vaccine mutations are unpublished data, Kelly. The drawing is not to scale.

ulent dengue 1 virus with amino acid substitutions in E residues 195 (domain I/II interface), 365 (domain I/III interface) and 405 (in the C-terminal stem), and NS3 438 had increased pathogenicity for mouse neuroblastoma cells and decreased infection efficiency for human hepatoma cells [26]. A chimeric dengue type 2 PDK-53/dengue 1 (C-prM-E) virus with mutations in E residues 130, 203 and 204 (domain I/II interface) and 225 (domain II) had an altered *in vitro* phenotype and reduced immunogenicity and neurovirulence in newborn mice [27]. A chimeric yellow fever–Japanese encephalitis (prM-E) virus with a single M279K mutation in the E domain I/II interface showed increased neurovirulence for mice and monkeys but decreased viscerotropism for monkeys [28]. A K204R domain I/II interface mutation in chimeric yellow fever-dengue 1 (prM-E) virus reduced neurovirulence for suckling mice and viremia for monkeys, and it was proposed that with the lysine to arginine change new intramolecular bonds may be formed among amino acids in E which could alter the pH requirement for E-fusion with the host cell membrane [29]. The other WRAIR PDK-passages vaccine candidates have at least one mutation in the domain I/II interface. Dengue 1 PDK20 has an E204K mutation, dengue 2 PDK50 has adjacent mutations E202K and N203D (Fig. 5) and dengue 3 PDK30 has mutation I195N (unpublished data, Zhao). Understanding how the mutations at the E domain interfaces affect virulence and growth during vaccine manufacture is highly relevant to development and safety testing of live, attenuated vaccines.

The other amino acid change in the dengue 4 E protein was at position 383 which was a single R by PDK6 and reverted to low passage parental mixture of G/R by PDK20. Dengue 4 residue 383 is located in domain III within a hairpin loop between beta strands F and G [19,30]. Mutations in this loop affected the antigenicity and immunogenicity of a dengue 3 virus [30]. Chimeric dengue 2/dengue 4 mutant viruses with amino acid substitutions at positions 383, 384 or 385 failed to react with and be neutralized by the monoclonal antibody 3H5 and exhibited reduced neurovirulence [31]. For tick-borne encephalitis virus, a monoclonal antibody escape mutant with substitution Y to H of amino acid 384 showed reduced pathogenicity in mice but retained its capacity to replicate and to induce a high-titered antibody response [32]. For a dengue 2 virus, residues 380–389 were involved in serotype-specific binding to mosquito but not mammalian cells [33]. The change from the mixture of G/R to R alone in PDK6 was not stable and may be an important mutation to isolate and evaluate for its potential to attenuate the virus.

A single amino acid E146Q substitution occurred in NS1. The NS1 protein is a conserved glycoprotein that is secreted from infected cells as a detergent-soluble hexamer [34]. Intracellular NS1 is multimeric and associated with intracellular and cell surface membranes and with other viral nonstructural proteins, and is also found located in sites of RNA synthesis [35,36]. The function of NS1 in biology of the virus is not fully understood, but it is believed to be involved in RNA replication [35–37]. There is evidence that NS1 is involved in antiviral immunity as it stimulates production of complement-fixing antibody and DHF/DSS patients have elevated levels of serum NS1 protein [38,39]. The E146Q NS1 mutation is not part of the C-terminal region required for dimerization [40]. The effect of the E146Q mutation is not known, but the significant charge change from acidic to hydrophilic may affect interactions of the NS1 protein with RNA or other proteins. The WRAIR dengue 1 and dengue 2 vaccine candidates each have a mutation in NS1, though at different positions (Fig. 5). For a dengue 2 16681 PDK53 vaccine strain, a G53D mutation in NS1 alone was responsible for temperature sensitivity, small-plaque phenotype, decreased replication in C6/36 cells and reduced neurovirulence in newborn mice [41].

Both NS4B mutations, S/L112L and A240V, are located in positions found for other flavivirus vaccine strains that have been adapted to grow in primate cells, and were determined for these viruses to be non-attenuating and even growth-enhancing [23,24,42,43]. Each of the WRAIR live-attenuated vaccine strains have a mutation in this region of NS4B (Fig. 5) and unpublished observation for dengue 3, results not shown (Bangti Zhao). NS4B is a small membrane-spanning hydrophobic protein with

a highly conserved hydrophobicity profile among flaviviruses but low sequence conservation. The NS4B protein is co-localized with the NS3 viral protease and helicase, and also with double-stranded RNA replication intermediates in cytoplasmic foci originating from the endoplasmic reticulum [44]. In *in vitro* experiments, NS4B was shown to dissociate NS3 from single-stranded RNA, suggesting that it is involved in RNA replication through enhancing NS3 helicase activity [45]. For dengue, West Nile and yellow fever viruses, NS4B was shown to inhibit interferon (IFN)-α/β signaling, and for dengue, the first 125 amino acids of NS4B were sufficient for this inhibition [46]. A membrane topology model for dengue NS4B localizes the N-terminal part of the protein (residues 1–100) in the endoplasmic reticulum lumen and identifies three C-terminal membrane-spanning domains with the C-terminal tail localized in the cytoplasm [44]. The S/L112L mutation is located within the first transmembrane domain, which together with the 2K fragment targets residues 1–100 to the ER lumen. The A240V mutation is located in the most C-terminal transmembrane domain, and the same mutation in a dengue 4 vaccine candidate was shown by others to be non-attenuating [42]. Knowledge of mutations relevant to enhancing virus growth vaccine cell substrates is important to producing live-attenuated or inactivated dengue virus vaccines.

The NS5 protein is the largest and most conserved of the flavivirus proteins. The NS5 mutation (F/L790L) occurred by PDK6 and is a conservative hydrophobic to aromatic change. The N-terminal region of the NS5 protein functions as a methyltransferase to help form the viral RNA type-1 cap, and the three-dimensional structure of the N-terminal methyltransferase domain has been determined [47,48]. The C-terminus of NS5 serves as an RNA-dependent RNA polymerase, and although the crystal structure of the flavivirus NS5 polymerase domain is not known, such information is available for the related hepatitis C virus, and multiple motifs and an interdomain have been assigned based on structural and functional studies [49,50]. To initiate replication of the single-stranded RNA, a small structural element of the polymerase serves as a scaffold for the 3′ end of the genome and copies the RNA in a primer-independent *de novo* manner [51,52]. This structural element is reported to have great variability in sequence among flaviviruses. For hepatitis C virus, mutations in this region along with other NS5 motif-mutations correlate with viral load and response to interferon treatment [53]. If the change from a mixture of F/L to L for residue 790 is attenuating, it may be related to the rate of initiation of RNA synthesis or to virus evasion of the innate immune response. It occurs by PDK6, along with the unstable E mutation of G/R383R and the presumed growth-adaptive NS4B A240V mutation.

Analysis of the mutations in the dengue 4 PDK20 virus suggests that only three amino acid changes, in E, NS1 and NS5, may be responsible for the attenuation of the vaccine strain in humans. These mutations will be isolated in future studies using the infectious clone and evaluated in assays that measure phenotypic differences. These are important steps toward understanding attenuation of flaviviruses, and toward developing alternate strategies to produce improved flavivirus vaccines.

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